Protein kinase A-mediated enhancement of miniature IPSC frequency by noradrenaline in rat cerebellar stellate cells

Satoru Kondo and Alain Marty

Max-Planck-Institut für biophysikalische Chemie, Arbeitsgruppe Zelluläre Neurobiologie, Am Faßberg, D-37077 Göttingen, Germany

1. Cellular mechanisms underlying the enhancement by noradrenaline (NA) of inhibitory postsynaptic currents (IPSCs) were studied at inhibitory synapses in the molecular layer of the cerebellum. IPSCs were obtained from stellate cells in rat cerebellar slices using tight-seal whole-cell recording.

2. Miniature IPSCs (mIPSCs) were recorded in the presence of tetrodotoxin (TTX; 100 nM). NA (10 μM) markedly increased the frequency of mIPSCs, but did not alter their mean amplitude. Bath application of the inhibitor of adenylyl cyclase 9-(tetrahydro-2′-furyl) adenine (SQ 22,536; 300 μM), of the wide spectrum protein kinase inhibitor staurosporine (1 μM), and of the R_P-diastereomer of adenosine-3′,5′-cyclic monophosphothioate (Rp-cAMPS; 500 μM), a specific inhibitor of cAMP-dependent protein kinase (PKA), inhibited the mIPSC frequency increase induced by NA.

3. The increase in mIPSC frequency was not attenuated by Cd^{2+} (100 μM), a blocker of voltage-dependent calcium channels. However, after a 12–15 min pre-incubation in Ca^{2+}-free saline, the effect of NA on mIPSCs was markedly inhibited. If Ca^{2+} ions were readmitted in the presence of NA, enhancement of the mIPSC frequency was largely restored.

4. Application of the membrane permeant analogue of cAMP, 8-Br-cAMP (1 mM), together with the inhibitor of cAMP phosphodiesterase, 3-isobutyl-1-methylxanthine (IBMX; 100 μM), caused a frequency increase of mIPSCs. Forskolin also mimicked the stimulatory effect of NA on mIPSC frequency. The effects of both 8-Br-cAMP and forskolin persisted in Ca^{2+}-free saline, suggesting that the modulation of transmitter release does not require Ca^{2+} influx.

5. On the whole, the results indicate that the potentiation of mIPSC frequency by NA is mediated through the sequential activation of adenylyl cyclase and protein kinase A (PKA), and that PKA modulates the vesicle release mechanism rather than Ca^{2+} influx. The lack of effect of NA after prolonged incubation in Ca^{2+}-free solution may be due to an inhibition of adenylyl cyclase by a gradual lowering of the cytosolic presynaptic Ca^{2+} concentration.

Noradrenaline (NA) exerts widespread modulatory actions in the brain. Noradrenergic fibres originate from the locus coeruleus and ventral lateral tegmentum, and they innervate many brain regions including the cerebral cortex, hippocampus and cerebellum (Ungerstedt, 1971). A large body of evidence shows that stimulation of both α- and β-adrenergic receptors alters the excitability of neurones by modifying voltage-dependent channels, as exemplified in hippocampal and sympathetic neurones (Gray & Johnston, 1987; Lipscombe, Kongsamut & Tsien, 1989). In addition, a number of studies describe modifications of synaptic strength by NA. At several synapses NA appears to augment the efficacy of transmission on a slow time scale. In the dentate gyrus, NA potentiates the field EPSPs elicited by perforant path stimulation (Lacaille & Harley, 1985). At the mossy fibre–CA3 synapse, NA increases the amplitude and duration of long-term potentiation (LTP), while the β-antagonist propranolol blocks LTP, suggesting a direct role of tetanus-released NA in the triggering of LTP (Hopkins & Johnston, 1988; Huang & Kandel, 1996). In cerebellar stellate and Purkinje cells, the frequency of spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs), resulting from the activity of presynaptic stellate and basket cells, is markedly enhanced by NA (Llano & Gerschenfeld, 1993*b*). The effects of NA at both CA3 and cerebellar synapses have a β-adrenergic pharmacological profile and can be mimicked by forskolin, suggesting that they are due to the activation of the cAMP pathway. In two other synapses where β-adrenergic effects are weak or absent, i.e. the inhibitory synapses onto

pyramidal CA3 neurones (Capogna, Gähwiler & Thompson, 1995) and the Schaffer collateral input onto pyramidal CA1 neurones (Chavez-Noriega & Stevens, 1994; Gereau & Conn, 1994), it has been shown that forskolin enhances synaptic transmission, suggesting that the cAMP pathway may have a widespread potentiating action on synaptic transmission.

Interestingly, it was clearly demonstrated that the potentiation induced by NA or forskolin is presynaptic in three of these preparations. In cerebellar synapses, neither the sensitivity of the postsynaptic cell to GABA nor the amplitude or kinetics of mIPSCs are modified, while the frequency of mIPSCs is enhanced (Llano & Gerschenfeld, 1993*b*). Likewise, the frequency, but not the amplitude, of miniature synaptic currents is enhanced at inhibitory synapses onto CA3 neurones and at excitatory synapses onto CA1 neurones (Chavez-Noriega & Stevens, 1994; Gereau & Conn, 1994; Capogna *et al.* 1995). The picture emerging from these studies is that a presynaptic elevation of cAMP enhances transmitter release at many central synapses and that NA uses this pathway when β-adrenergic receptors are present in the presynaptic membrane.

It is a considerable challenge to identify the intracellular presynaptic mechanisms responsible for cAMP-induced potentiation in a slice preparation. Capogna *et al.* (1995) showed that a blocker of protein kinase A (PKA) abolishes the effects of forskolin and concluded that these effects are due to the activation of PKA. The same authors also showed that the forskolin-induced potentiation is preserved in the presence of Cd^{2+}, indicating that it does not involve any regulation of voltage-dependent Ca^{2+} channels. Likewise, Llano & Gerschenfeld (1993*b*) suggested that the NA-induced potentiation of miniature frequency in the cerebellum does not depend on regulation of Ca^{2+} entry, because the control rate of mIPSCs (without NA) was not affected by Cd^{2+}. However, the effects of NA were not tested in the cerebellum under conditions where Ca^{2+} channels were blocked.

The aim of the present study was to investigate the mechanisms underlying NA effects in cerebellar interneurones, and in particular to test whether the cAMP pathway is involved. Along the way unexpected results were obtained concerning the Ca^{2+} sensitivity of the NA effect.

METHODS

Sagittal cerebellar slices (180 μm in thickness) were prepared as previously described (Llano & Gerschenfeld, 1993*a*) from 13- to 21-day-old rats that were decapitated following cervical dislocation. Tight-seal whole-cell recordings were made from visually identified stellate cells located in the upper two-thirds of the molecular layer.

Whole-cell recording pipettes (3–4 MΩ) were pulled from borosilicate glass and filled with a solution containing (mM): 120 KCl, 4·6 $MgCl_2$, 10 EGTA, 1 $CaCl_2$, 10 Hepes, 4 Na-ATP and 0·4 Na-GTP; pH adjusted to 7·3 with KOH. The chamber was perfused at a rate of 1–1·5 ml min^{-1} with a solution containing (mM): 125 NaCl, 2·5 KCl, 1·25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$ and 10 glucose, equilibrated with a 95% O_2 and 5% CO_2 mixture. The osmolality of these solutions was in the range 295–305 mosmol kg^{-1}. All recordings were made at room temperature (20–25 °C). DL-2-Amino-5-phosphonovaleric acid (APV; 25 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), were routinely included in the recording solution in order to block ionotropic glutamatergic transmission.

Whole-cell recordings were performed while visualizing the cells with a ×63 water immersion lens. Typical values for the series resistance during whole-cell recording were 10–15 MΩ.

Data acquisition and analysis were performed as previously described (Llano & Gerschenfeld, 1993*a*). Results are expressed as means ± S.E.M., and the significance of the results was assessed using Student's *t* test. Control experiments (effects of NA under normal conditions) were systematically alternated with test experiments, so that each series of experiments had its own set of control measurements.

Pharmacological agents were applied by bath application and reached their steady-state concentration in the chamber (volume 700 μl) within 2 min. APV and CNQX were purchased from Tocris Cookson, Bristol, UK. (−)-Noradrenaline (NA) bitartrate, forskolin, the R_P-diastereomer of adenosine-3′,5′-cyclic monophosphothioate (Rp-cAMPS) and staurosporine were purchased from Research Biochemicals International. SQ 22,536 was a generous gift from the Bristol-Myers Squibb Corporation (Princeton, NJ, USA). All other chemicals used were purchased from Sigma. Stock solutions were prepared as follows: forskolin and staurosporine were dissolved in dimethylsulphoxide (DMSO) at 10 mM; tetrodotoxin (TTX) was dissolved in 0·5% v/v acetic acid at 200 μM; NA was prepared at 10 mM in water. These stock solutions were frozen, and final concentrations were obtained by dilution in saline just before use.

RESULTS

NA increases the mIPSC frequency

Figure 1 illustrates the effect of NA on miniature synaptic activity of a stellate cell. Figure 1*Aa* and *b* display continuous recordings of mIPSCs in control and NA-containing saline, respectively. The trace in Fig. 1*Ab*, which was taken 3 min after applying NA to the external fluid, shows that NA caused a marked increase of synaptic activity. The frequency of mIPSCs increased, while their mean amplitude or kinetics of decay remained unchanged. This effect was observed within a few minutes after addition of NA to the bath solution, but recovery upon washout was slow (over 20 min).

Staurosporine inhibits the effect of NA

Llano & Gerschenfeld (1993*b*) have established that NA exerts its potentiating effects through the activation of β-adrenergic receptors. It seems likely, therefore, that the next steps in the transduction pathway consist of activation of adenylyl cyclase and of cAMP-dependent protein kinase A (PKA). Other schemes are, however, possible; for instance, cAMP could act directly on a target molecule such as an ion channel (Pedarzani & Storm, 1995). As a test for the involvement of a protein kinase, the effects of the broad spectrum protein kinase inhibitor staurosporine were investigated. Staurosporine (1 μM) was added to the perfusion chamber 15 min before the experiment was

performed. In the presence of staurosporine, NA increased the mIPSC frequency by 159 ± 22% ($n = 6$), a ratio significantly lower than that observed in control saline (239 ± 21%, $n = 6$) ($P < 0{\cdot}02$; Figs 1*B* and 2). Staurosporine itself had no effect on the frequency or on the mean amplitude of mIPSCs. In staurosporine, mean amplitude was not affected significantly by application of NA: on average the mean amplitude in the presence of NA was 116 ± 11% ($n = 6$) of control (Fig. 2).

The enhancement of mIPSC frequency by NA is sensitive to inhibitors of adenylyl cyclase and PKA

If cAMP mediates the effects of NA, inhibition of adenylyl cyclase activity should reduce the action of NA. Application of 9-(tetrahydro-2-furyl) adenine (SQ 22,536; 300 μM), an agent that is known to decrease the activity of adenylyl cyclase (Madison & Nicoll, 1986), reduced the action of NA on the mIPSC frequency (Fig. 3*A*). The cerebellar slice was pre-incubated in the same concentration of SQ 22,536 for

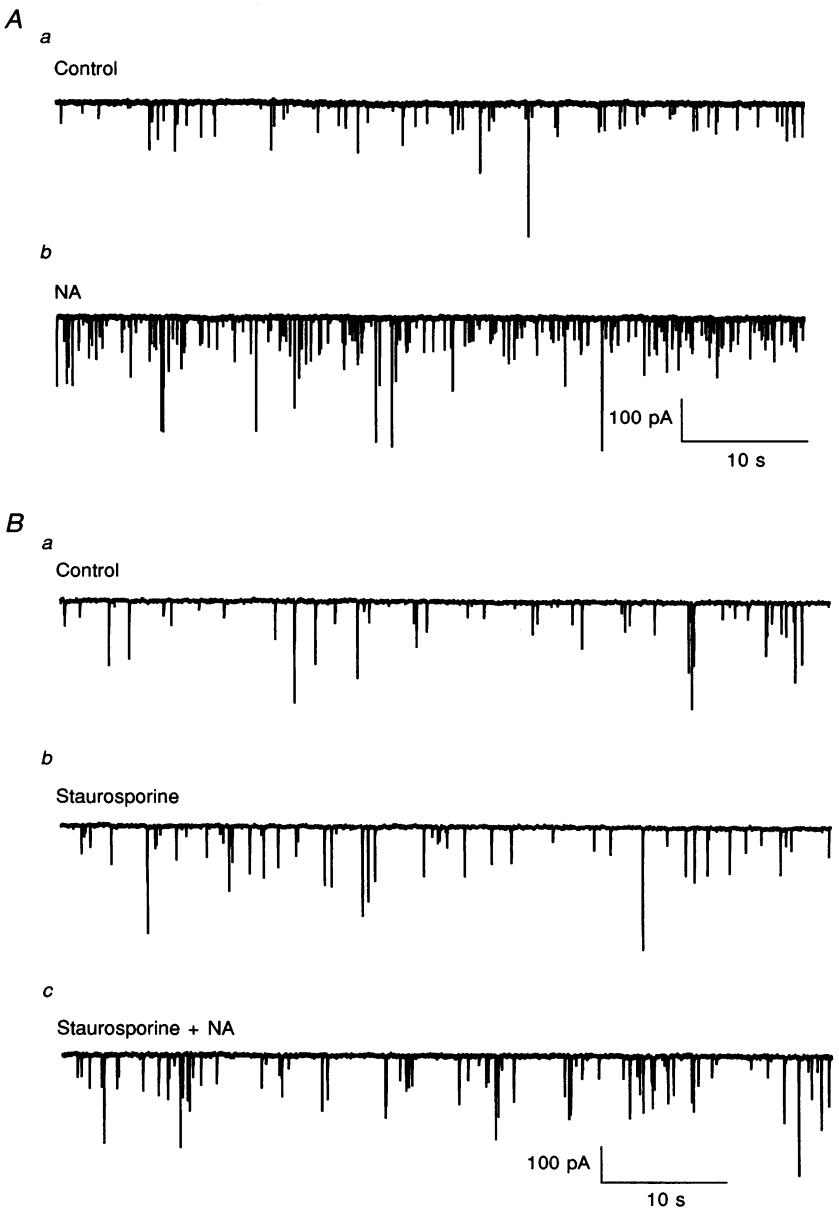

Figure 1. NA-induced increase in mIPSC frequency in control saline and in the presence of staurosporine

A, noradrenaline (10 μM) enhances the frequency of mIPSCs in stellate cells; continuous recordings (1 min duration) of mIPSCs in control conditions (*a*) and in the presence of NA (*b*). Both records were obtained from the same cell; TTX (0·1 μM) was present throughout. *B*, recordings (1 min duration) of mIPSCs in control conditions (with TTX; *a*), after addition of 1 μM staurosporine (*b*), and after further addition of 10 μM NA (*c*). All records from the same cell. The cell in *B* is different from that in *A*. Staurosporine had no effect on mIPSCs when applied alone, but it reduced the NA-induced increase in mIPSC frequency.

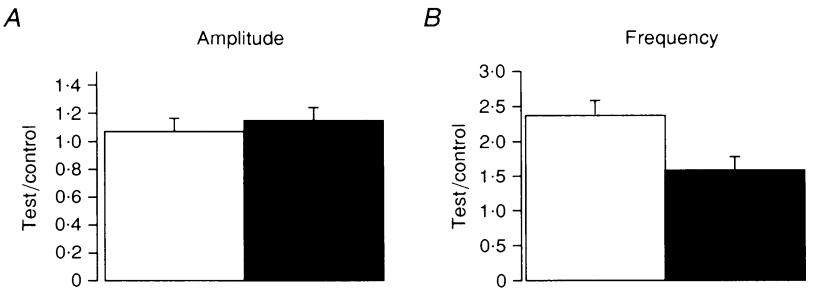

Figure 2. Comparison of the effects of NA between untreated and staurosporine-treated cells

Pooled data from three untreated cells (□) and six staurosporine-treated cells (■), as in Fig. 1*A* and *B*, respectively. The ratios of mean amplitudes (*A*) and frequencies (*B*) obtained in 10 μM NA over control values (either in normal saline or in the presence of staurosporine) are indicated, together with the corresponding standard error of the mean. The mean mIPSC amplitude was not altered by NA in either normal saline or staurosporine. The increase of mIPSC frequency induced by NA in staurosporine is significantly less than in untreated slices ($P < 0{\cdot}02$).

periods of 30 min (five experiments) or 5 h (two experiments). Control experiments were performed alternately with SQ 22,536 experiments; slices were then kept in the experimental chamber in normal saline for the same time. Significant inhibition of the NA response was obtained both with 30 min and 5 h pre-incubation. In pooled data from the two incubation times, the mIPSC frequency increase was drastically reduced upon SQ 22,536 treatment: in the control experiments NA increased the frequency to 216 ± 29% of the control ($n = 5$), while in the presence of the inhibitor, the increase was 124 ± 10% of control ($n = 7$; $P < 0{\cdot}02$; Fig. 3*A*). These data were all collected 3–5 min after switching to the NA solution. In the control, this corresponds to the peak of the NA effect (Llano &

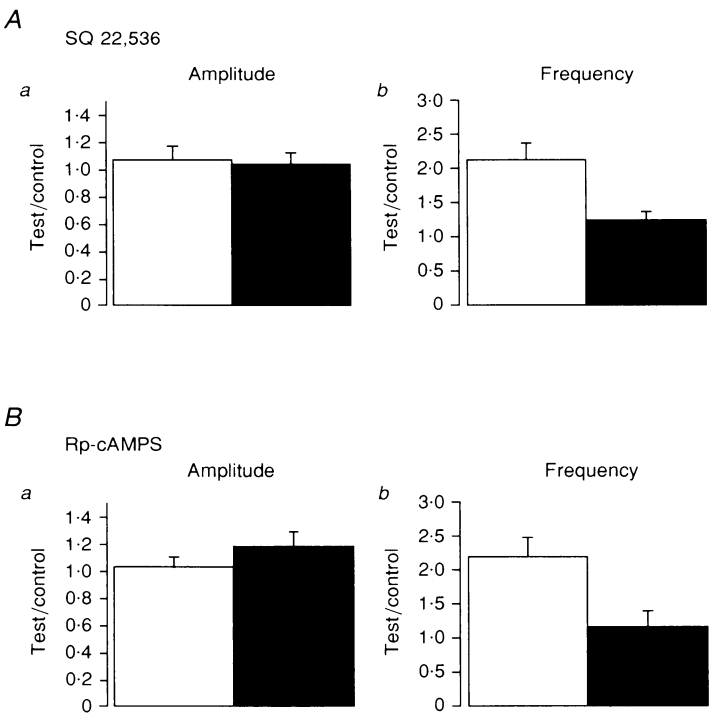

Figure 3. The effects of SQ 22,536 and Rp-cAMPS on the increase of mIPSC frequency by NA

A, pooled data from seven SQ 22,536-treated (■) and five untreated cells (□). The increase of mIPSC frequency was strongly inhibited by the application of SQ 22,536 ($P < 0{\cdot}02$) (*b*). The mean mIPSC amplitude was not altered by SQ 22,536 and/or NA (*a*). *B*, pooled data from five Rp-cAMPS-treated (■) and five untreated cells (□). Rp-cAMPS significantly reduced the synaptic potentiation induced by NA ($P < 0{\cdot}05$) (*b*). Mean mIPSC amplitude did not change as a result of either Rp-cAMPS pre-incubation or NA exposure (*a*). In all graphs the control measurements correspond to values obtained before the addition of NA; thus these values are in normal saline for the 'untreated' data, and in Rp-cAMPS and SQ 22,536 for the 'Rp-cAMPS-treated' and 'SQ 22,536-treated' data, respectively.

Gerschenfeld, 1993*b*). In the presence of SQ 22,536 however, it was noticed that the mIPSC frequency slowly grew with time upon prolonged application of NA, so that data collected at 9–15 min in NA gave an enhancement of mIPSC frequency of 208 ± 28% ($n = 7$) of control. Therefore SQ 22,536 appears to delay the development of the NA effect. The mean of the mIPSC amplitudes was not altered significantly by NA in the presence of SQ 22,536 (105 ± 6% of control, $n = 7$). SQ 22,536 itself had no significant effect on mIPSC amplitude (105 ± 5% of control, $n = 5$), but did increase their frequency somewhat (131 ± 19% of control, $n = 5$). These results show that inhibiting the adenylyl cyclase diminishes markedly the NA-induced increase in mIPSC frequency.

Staurosporine is a broad spectrum protein kinase inhibitor; affected kinases include PKA and PKC (Rüegg & Burgess, 1989). In order to determine which protein kinase pathway was responsible for the effects of staurosporine on IPSC regulation, we investigated the effects of the specific inhibitor of PKA, Rp-cAMPS, a non-hydrolysable cAMP analogue. Rp-cAMPS has the disadvantage that it is less readily diffusible than staurosporine in brain tissue. Slices were therefore incubated in the presence of Rp-cAMPS at a concentration of 500 μM for 3–5 h. In these slices NA failed, as a rule, to increase the mIPSC frequency (118 ± 23% of control, $n = 5$; Fig. 3*B*). However, in one of these five experiments we observed an increase in mIPSC frequency to 209% of control, perhaps reflecting poor access of Rp-cAMPS to presynaptic terminals. In control experiments, where slices were incubated in normal saline for the same time, NA increased the mIPSC frequency to 224 ± 23% of the control ($n = 5$) (Fig. 3*B*). The inhibition linked to Rp-cAMPS incubation was statistically significant ($P < 0.05$). The mean mIPSC amplitude was not altered by NA in these experiments, with a mean amplitude of 107 ± 14% ($n = 5$) of control in Rp-cAMPS, and of 102 ± 10% ($n = 5$) of control in the long saline incubation experiments. Contrary to the results in SQ 22,356, the inhibition by Rp-cAMPS (or the above effects by staurosporine) could not be overcome by increasing the exposure time to NA.

8-Br-cAMP increases the frequency of mIPSCs

As a further test of the involvement of cAMP, the effects of the membrane-permeable cAMP analogue, 8-Br-cAMP

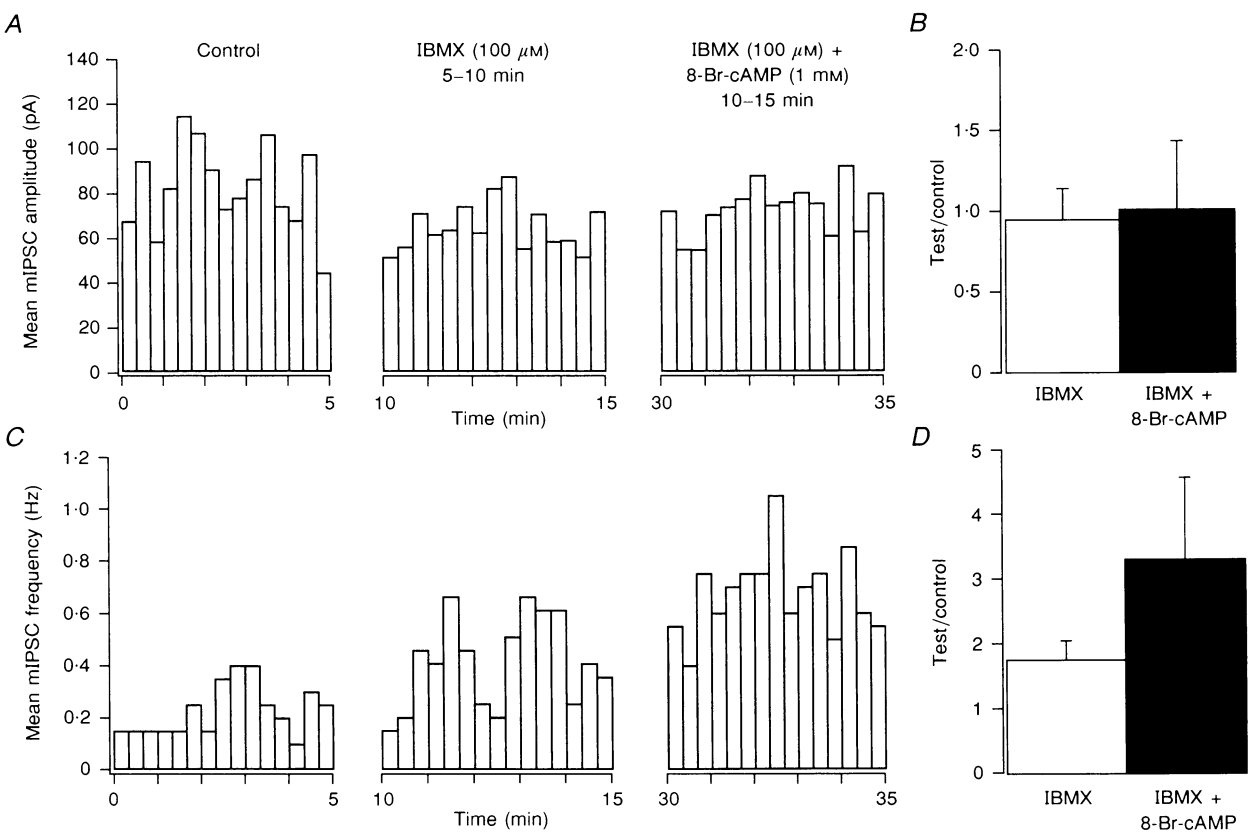

Figure 4. Increase in mIPSC frequency following application of 8-Br-cAMP

A and *C* show plots against time of mean amplitude (*A*) and mean frequency (*C*) of mIPSCs averaged over 1 min time periods. The slice was perfused with IBMX (100 μM) for 15 min before 8-Br-cAMP (1 mM) was added. *B* and *D* show pooled data from four cells in IBMX, and 8-Br-cAMP in the presence of IBMX. Control values refer to the measurements performed in standard TTX-containing saline at the beginning of each experiment. IBMX increased the mIPSC frequency, and a further increase was observed in 8-Br-cAMP in the continuous presence of IBMX.

(1 mM), were investigated. These experiments were performed in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 100 μM) to prevent the degradation of the cAMP analogue. IBMX was applied to the perfused saline 15 min before 8-Br-cAMP. This resulted in a significant increase in mIPSC frequency (176 $\pm$ 25% of control, $n = 4$) but did not lead to any significant change of the mean mIPSC amplitude (95 $\pm$ 16% of control, $n = 4$; Fig. 4). Following 8-Br-cAMP application in the continuous presence of IBMX, the mIPSC frequency was increased further to 328 $\pm$ 126% of the initial control (in normal TTX-containing saline) and to 198 $\pm$ 119% of the IBMX value ($n = 4$; Fig. 4). The mean amplitude of mIPSCs in 8-Br-cAMP was 101 $\pm$ 40% of the initial control and 109 $\pm$ 51% of the IBMX value ($n = 4$; Fig. 4). These results indicate that the cAMP analogue is able to bypass the β-adrenergic receptor to modulate the mIPSC frequency.

The NA-induced increase in mIPSCs does not depend on activation of voltage-sensitive Ca^{2+} channels

It was recently demonstrated that isoprenaline increases synaptic transmission in the amygdala by enhancing the opening probability of specific subtypes of voltage-sensitive Ca^{2+} channels (Huang, Hsu & Gean, 1996). If the augmentation of mIPSC frequency was due to such a mechanism, it should be abolished by blocking voltage-sensitive Ca^{2+} channels. We therefore tested the effectiveness of NA after adding Cd^{2+} to the saline. As previously reported, the mean amplitude of the mIPSCs was slightly reduced in Cd^{2+}-containing saline but the frequency of events

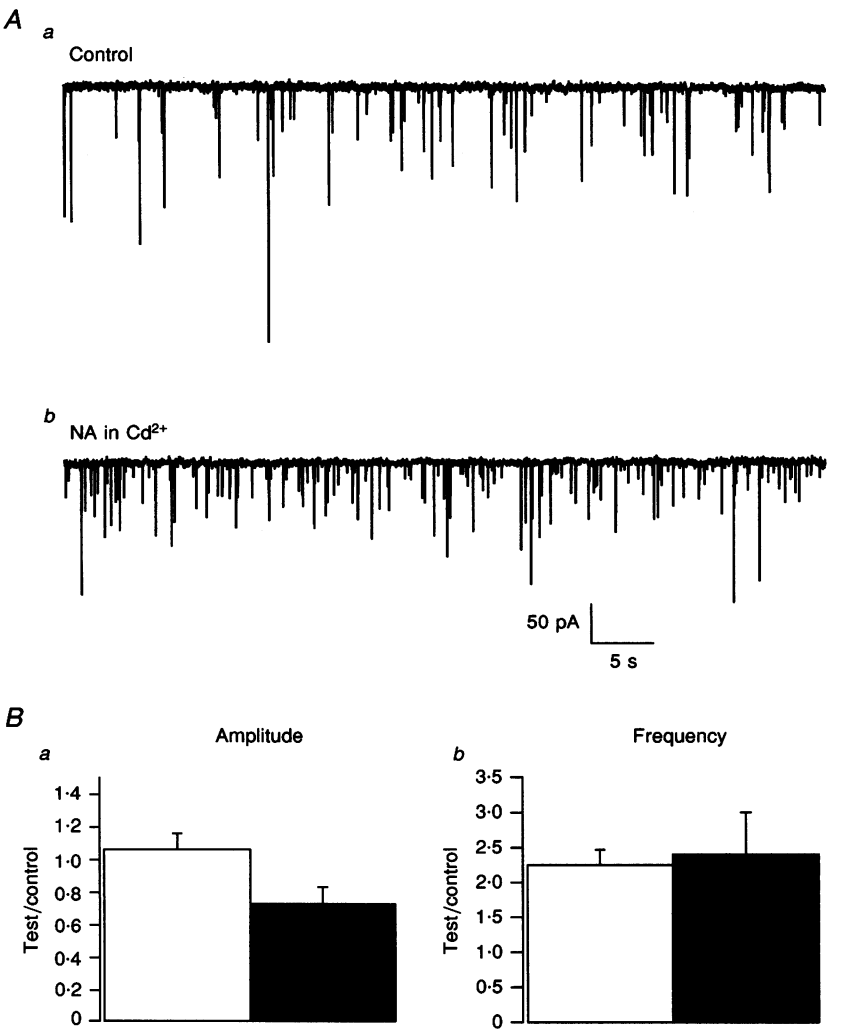

Figure 5. Voltage-gated Ca^{2+} channels are not involved in the effects of NA

Cd^{2+} (100 μM) has no effect on the NA-induced increase in mIPSC frequency. *A*, 1 min continuous recording under control conditions (*a*), and in the presence of NA and Cd^{2+} (*b*), from the same cell. NA was added after 5 min of Cd^{2+} application. The mean frequency of mIPSCs is increased in *b* while their mean amplitude is decreased. *B*, pooled data from five cells in normal saline (□) and five cells in Cd^{2+} saline (■). Control values refer to the measurements performed in standard TTX-containing saline at the beginning of each experiment. Cd^{2+} reduced the mean amplitude of mIPSCs (*a*). NA was able to increase the mIPSC frequency to the same extent in normal saline, and in the presence of Cd^{2+} (*b*).

was not affected (Llano & Gerschenfeld, 1993*a*). In the presence of Cd^{2+}, as in control saline, the mean amplitudes of mIPSCs were similar before and after application of NA (73 ± 13% ($n = 5$) and 68 ± 10% ($n = 5$) of the mean IPSC amplitude in control saline, respectively). The lower amplitude of mIPSCs observed in the presence of Cd^{2+} is likely to reflect the blocking action of Cd^{2+} on GABA channels (Kaneko & Tachibana, 1986). Application of NA in the presence of Cd^{2+} increased the frequency of mIPSCs to 242 ± 50% ($n = 5$) of control, which was not different from the increase obtained in the absence of Cd^{2+} (227 ± 22%, $n = 5$; $P > 0{\cdot}8$; Fig. 5*Bb*). We conclude from these experiments that the action of NA on mIPSCs does not require Ca^{2+} influx into the presynaptic terminal through voltage-dependent Ca^{2+} channels.

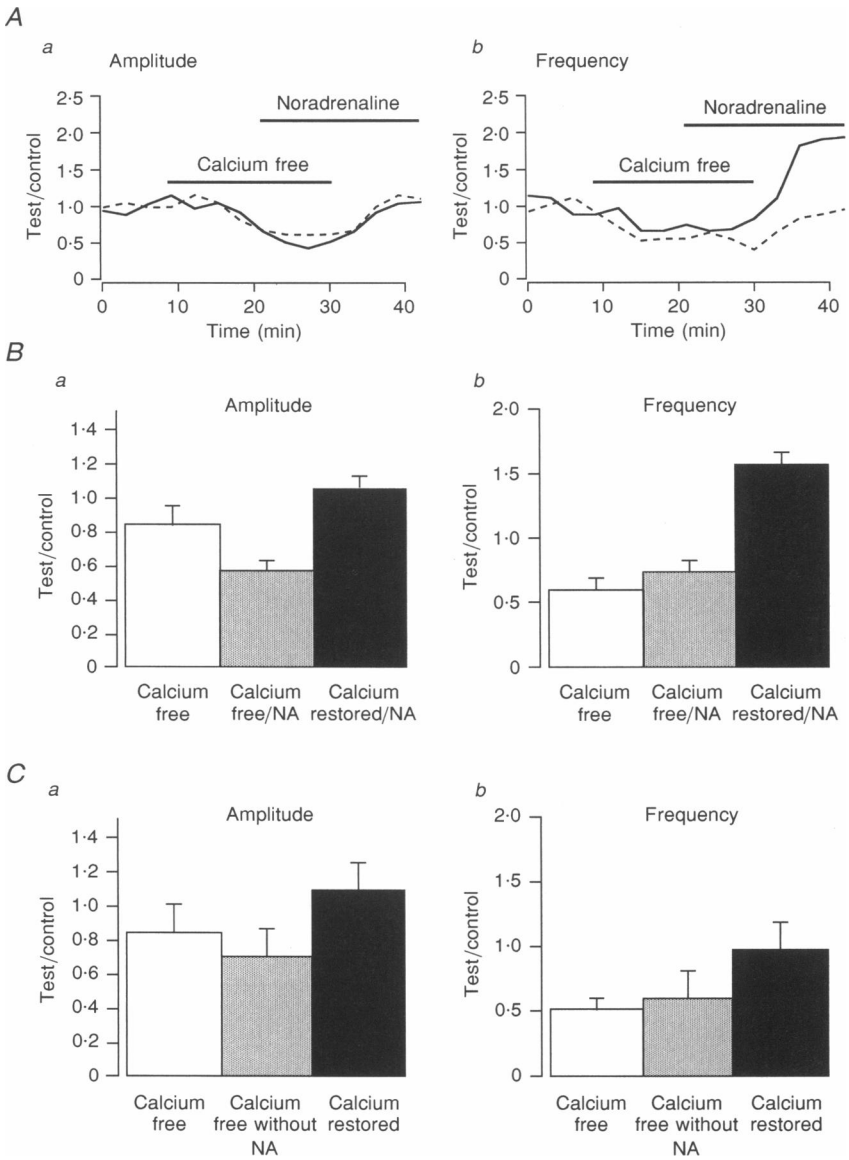

Figure 6. Lack of NA effect in Ca^{2+}-free saline

A, plots against time of mean amplitude and mean frequency of mIPSCs averaged over 3 min time periods. The slice was perfused with Ca^{2+}-free saline containing 200 μM EGTA for 12 min before NA was added. After 9 min in NA in Ca^{2+}-free saline, Ca^{2+} was restored in the continuous presence of NA (continuous line). The plot of a reference experiment, from a different cell is also shown; the cell was simply treated with Ca^{2+}-free saline for the same time period without NA, and then re-exposed to Ca^{2+} (dashed line). *B*, pooled data from six cells. Pre-exposure to the Ca^{2+}-free saline was for either 12 or 15 min, otherwise the protocol was as in *A*. NA failed to increase the mIPSC frequency in Ca^{2+}-free saline. However, by adding Ca^{2+} back to the recording chamber, NA recovered some of its potentiating effects. *C*, pooled data of reference experiments from four cells. The results are similar to those of NA-treated cells above, except for the increase of mIPSC frequency after readmission of Ca^{2+}. Control values refer to the measurements made in standard TTX-containing saline at the beginning of each experiment.

Changes in extracellular Ca^{2+} concentration affect the action of NA on mIPSCs

The results described above indicate that modifications of voltage-dependent Ca^{2+} channels cannot account for the enhancement of mIPSC frequency by NA. However, NA could modify Ca^{2+} influx through some other conductance pathway that would not be blocked by Cd^{2+}. To investigate this possibility, experiments were performed in Ca^{2+}-free, EGTA-containing saline. After 12–15 min exposure to a Ca^{2+}-free solution, the frequency of mIPSCs was decreased to $62 \pm 7\%$ ($n = 6$) of control, while the amplitude was slightly reduced ($84 \pm 12\%$ of the control, $n = 5$; Fig. 6*A* and *Ba*). If at this stage NA was added to the perfusion chamber, still maintaining the external solution Ca^{2+} free, no significant increase in the mIPSC frequency was observed (Fig. 6*A* and *Bb*). On the other hand, the mean amplitude of the mIPSCs decreased further in the Ca^{2+}-free solution containing NA (to $57 \pm 6\%$ of the initial control, $n = 6$). Simply exposing slices to a Ca^{2+}-free saline for a long time period induced a continuous decrease in mIPSC amplitude (to $72 \pm 17\%$, $n = 5$; Fig. 6*Ca*), similar to that observed in the Ca^{2+}-free solution with NA. Therefore the amplitude decrease is due to the lowering of the extracellular Ca^{2+} concentration and is independent of the presence of NA. By re-adding 2 mM Ca^{2+} to the NA-containing solution, a marked potentiation of the mIPSC amplitude was observed, presumably as a result of restoring the normal internal Ca^{2+} concentration. The frequency of mIPSCs was increased to $160 \pm 7\%$ ($n = 6$) of the control (Fig. 6*A* and *Bb*). This is a significant increase, even though it is less marked than the $221 \pm 17\%$ ($n = 10$) increase observed without Ca^{2+}-free pre-incubation. Furthermore, this value is significantly higher than the value ($98 \pm 24\%$, $n = 5$) obtained with cells that were simply exposed to the Ca^{2+}-free saline and then re-exposed to 2 mM Ca^{2+}. The mean amplitude also recovered upon readmission of 2 mM Ca^{2+} ($106 \pm 8\%$ of control, $n = 6$; Fig. 6*A* and *Ba*).

In summary, these experiments show that NA loses its ability to influence the mIPSC frequency after incubation in a Ca^{2+}-free solution, but that this ability can be partially restored by readmission of Ca^{2+}.

8-Br-cAMP and forskolin increased the frequency of mIPSCs in Ca^{2+}-free saline

One interpretation for the failure of NA to influence mIPSC frequency in Ca^{2+}-free saline, could be that the effect of NA on mIPSCs requires Ca^{2+} influx through a permeation pathway that is not blocked by Cd^{2+}. Alternatively, the decrease in the presynaptic cytosolic Ca^{2+} concentration, which undoubtedly accompanies long exposure to the Ca^{2+}-free solution, could have resulted in some disruption of the second messenger pathway involved in the NA response, indicating that both the presynaptic Ca^{2+} concentration and β-receptor activation contribute to the stimulation of adenylyl cyclase. In order to decide between these alternative possibilities, we tested the ability of 8-Br-cAMP

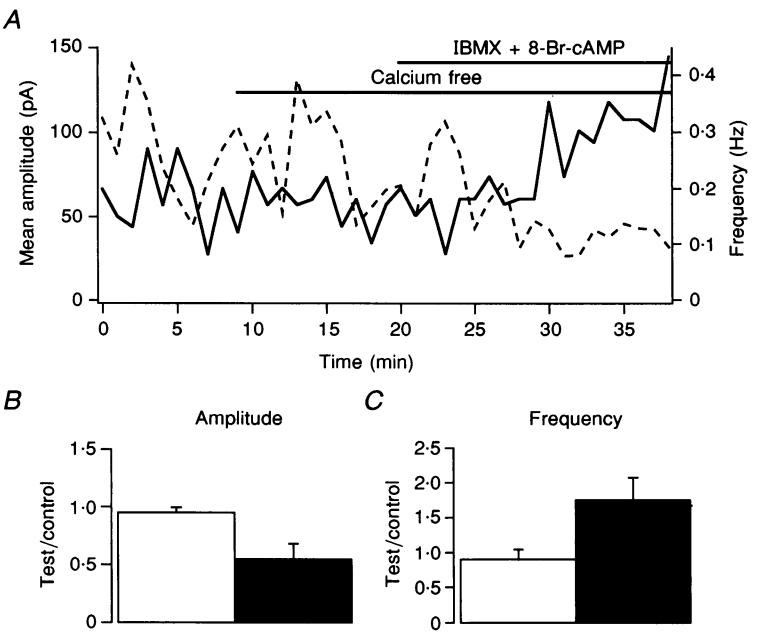

Figure 7. Persistent effectiveness of 8-Br-cAMP on mIPSC frequency in Ca^{2+}-free saline

A, plots against time of mean amplitude (dashed line) and mean frequency (continuous line) of mIPSCs averaged over 1 min time periods. The slice was perfused with Ca^{2+}-free saline containing 200 μM EGTA for 12 min, before 8-Br-cAMP (1 mM) and IBMX (100 μM) were added. *B* and *C*, pooled data from three cells. 8-Br-cAMP in the presence of IBMX increased the mIPSC frequency in Ca^{2+}-free saline. Control values refer to the measurements performed in standard TTX-containing saline at the beginning of each experiment. □, calcium free (12 min); ■, 8-Br-cAMP + IBMX/calcium free.

and forskolin to potentiate GABA release in Ca^{2+}-free saline. For the 8-Br-cAMP experiments, the slices were first exposed over 12 min to the Ca^{2+}-free solution to match the conditions of the NA experiments (Fig. 6). 8-Br-cAMP (1 mM) was added with IBMX (100 μM) to the perfusion chamber at this stage, still maintaining the external solution Ca^{2+} free. This resulted in a significant increase in the mIPSC frequency to 180 ± 28% of the initial control value and 195 ± 12% of the Ca^{2+}-free value ($n = 3$; Fig. 7*A* and *C*). These results indicate that 8-Br-cAMP retains the ability to increase mIPSC frequency after incubation in Ca^{2+}-free solution. In the presence of 8-Br-cAMP, the mean amplitude was decreased to 55 ± 11% of the initial control value and 59 ± 10% of the Ca^{2+}-free value ($n = 3$). However, as in the experiments with NA described above, and in those with forskolin described below, this probably reflects the effect of prolonged incubation in the Ca^{2+}-free solution rather than a genuine effect of 8-Br-cAMP.

Forskolin (20 μM) strongly increases the frequency of mIPSCs in normal saline (Llano & Gerschenfeld, 1993*b*). In the forskolin experiments, the time of exposure to the Ca^{2+}-free, EGTA-containing solution was extended to 47–70 min. After such long incubations in Ca^{2+}-free saline, the frequency of mIPSCs was markedly decreased and the mean amplitude of mIPSCs was about half of the control value (Fig. 8). These effects are presumably presynaptic since the postsynaptic Ca^{2+} concentration was strongly buffered in these experiments with a 10 mM BAPTA internal solution.

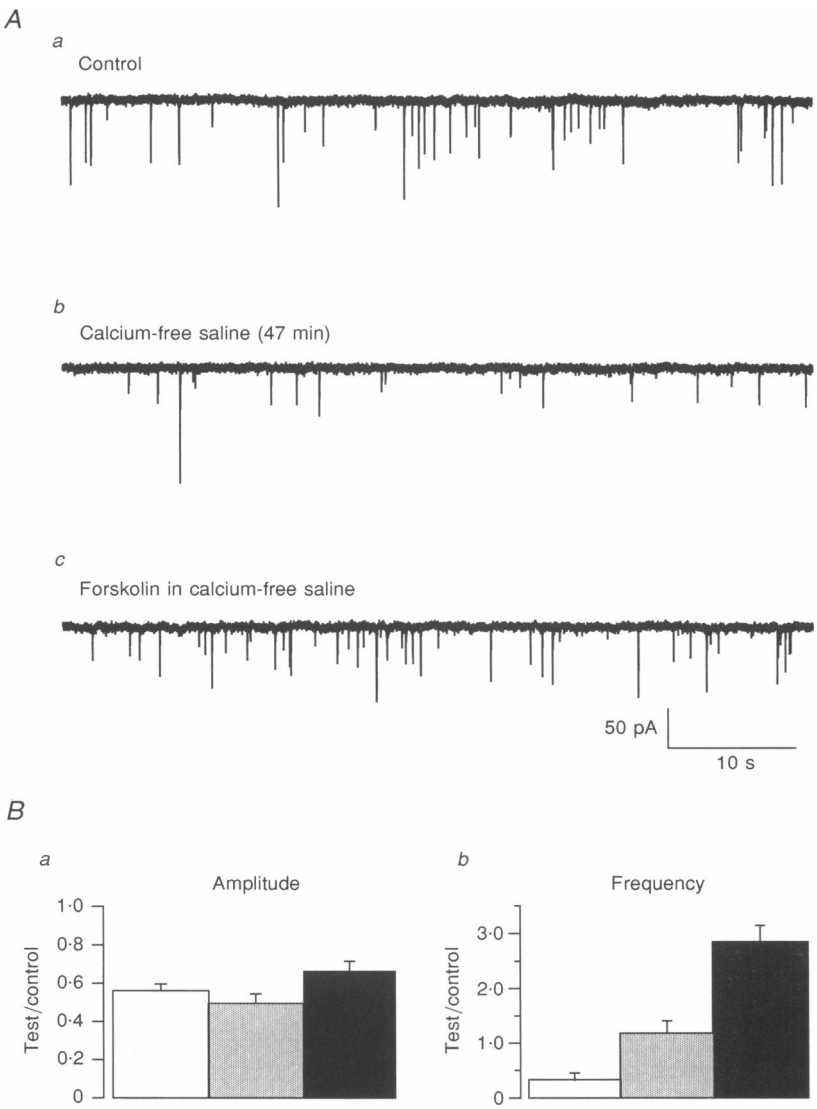

Figure 8. Persistent effectiveness of forskolin on mIPSC frequency in Ca^{2+}-free saline

A, 1 min continuous recordings comparing mIPSCs obtained in control TTX-containing saline (*a*), then in Ca^{2+}-free saline containing TTX and 200 μM EGTA (*b*), and finally after addition of 20 μM forskolin to the Ca^{2+}-free, TTX-containing saline (*c*). *B*, pooled data from four cells. Forskolin was still able to increase the frequency of mIPSC in Ca^{2+}-free saline. Control values refer to the initial period in TTX-containing saline. □, calcium-free (47–70 min); ▨, calcium free/forskolin; ■, calcium restored/forskolin.

The long incubation in Ca^{2+}-free saline did not appear to damage the cells, which retained a normal aspect together with a stable holding current and series resistance throughout the experiment. Furthermore, if Ca^{2+} ions were readmitted to the bath after a long incubation in Ca^{2+}-free saline, the frequency of mIPSCs recovered to a value close to the control level (data not shown, $n = 2$).

After 47–70 min pre-incubation in Ca^{2+}-free saline the enhancement by forskolin (20 μM) was still observed, resulting in an increase in mIPSC frequency to 120 ± 20% of the initial control (in normal saline) and to 426 ± 75% of the Ca^{2+}-free value ($n = 4$; Fig. 8). This last ratio was almost the same as the ratio obtained if the entire experiment was conducted in normal saline (473 ± 63%, $n = 4$). In the combined presence of forskolin and the Ca^{2+}-free solution, the mean mIPSC amplitude was still decreased compared with the normal saline value (by 50 ± 6%, $n = 4$; Fig. 8).

Overall, the results obtained with 8-Br-cAMP and with forskolin do not support the hypothesis that PKA-dependent enhancement requires Ca^{2+} influx. They suggest instead that a drop in the cytoplasmic Ca^{2+} concentration influences the second messenger pathway involved in the NA effect.

DISCUSSION

NA acts through the adenylyl cyclase–PKA pathway

The present finding that SQ 22,536 inhibits the effects of NA on mIPSCs indicates that NA is coupled to cAMP production. This conclusion is in line with the previous pharmacological characterization by Llano & Gerschenfeld (1993*b*) of the response as β-adrenergic. The fact that staurosporine and Rp-cAMPS strongly inhibit the response provides convincing evidence that cAMP then acts through activation of PKA. Moreover, application of the cAMP analogue 8-Br-cAMP increased the frequency of mIPSC and mimicked the effect of NA. It can be concluded that the effects of NA on mIPSC frequency involve sequentially the activation of adenylyl cyclase, cAMP production and activation of PKA.

Since neither SQ 22,536 nor staurosporine decreased the mIPSC frequency prior to NA application, it appears that the PKA-regulated pathway is not active in unstimulated preparations.

The target of PKA remains a mystery. Because the potentiating effect of cAMP is observed in many different preparations, and because it does not appear to involve the regulation of Ca^{2+} influx (see Introduction, and Discussion below), it is tempting to hypothesize that PKA phosphorylates one of the proteins involved in the mobilization, docking or fusion of synaptic vesicles. Several such proteins, including synapsins I and II, can be phosphorylated by PKA, but the exact functional significance of the phosphorylation state of these proteins is presently unknown (Greengard, Valtorta, Czernic & Benfenati, 1993).

NA-induced potentiation is not due to modulation of Ca^{2+} influx

This is indicated by two main lines of evidence: (i) in the presence of Cd^{2+} ions, all voltage-dependent Ca^{2+} channels should be blocked, and yet the effect of NA on mIPSC frequency is as pronounced as in control saline; (ii) even after prolonged incubation in Ca^{2+}-free saline, 8-Br-cAMP and forskolin are able to enhance the rate of mIPSCs. These results confirm the conclusions reached by Llano & Gerschenfeld (1993*b*) based on more indirect evidence. They also reinforce the parallels noted in the Introduction between the effects of forskolin in stellate cells and CA3 pyramidal cells (Capogna *et al.* 1995).

Effects of long incubation in Ca^{2+}-free saline

After incubation in Ca^{2+}-free saline, the action of NA is markedly attenuated, while the activator of adenylyl cyclase, forskolin, has effects comparable to those observed in normal Ca^{2+}. It is widely known that Ca^{2+} influences the level of cAMP through the ability of the calcium–calmodulin complex to activate adenylyl cyclase (reviewed in Cohen, 1988, and in Cooper, Mons & Karpen, 1995). Moreover, molecular cloning experiments have uncovered eight subtypes of adenylyl cyclase so far and intensive biochemical experiments suggest that they are multiply regulated; for certain subtypes, Ca^{2+}, PKC and the $\beta\gamma$-subunit of G proteins are more powerful modulators than the α-subunits (Cooper *et al.* 1995). According to *in situ* hybridization analysis, three types of adenylyl cyclase are expressed in the cerebellum in significant amounts, namely types I, II and VII (Cooper *et al.* 1995). From biochemical studies, type I adenylyl cyclase is strongly stimulated by Ca^{2+} and mildly by G protein activation, and this enzyme is stimulated synergistically by Ca^{2+} and receptor activation (Wayman, Impey, Wu, Kindsvogel, Prichard & Storm, 1994), while types II and VII are not sensitive to Ca^{2+} (Cooper *et al.* 1995). In view of these results, we propose that the subtype of adenylyl cyclase responsible for the stimulatory effects of NA in cerebellar interneurones is Ca^{2+} sensitive, possibly type I. In such a case the weakness of the action of NA in Ca^{2+}-free solution would be a consequence of the inhibition of this adenylyl cyclase subtype following a drop in the internal Ca^{2+} concentration. Indeed, 8-Br-cAMP bypassed the effect of NA in Ca^{2+}-free saline and this result suggests that both Ca^{2+} and β-receptor activation co-operate to produce the cAMP involved in the frequency increase of mIPSCs. The exact reason why forskolin effects are not affected is presently unclear. Because a forskolin analogue that does not activate the cAMP–PKA cascade did not enhance the mIPSC frequency (Llano & Gerschenfeld, 1993*b*), it is likely that forskolin effects are mediated by adenylyl cyclase. It may be that the sensitivity of the cyclase to the internal Ca^{2+} concentration depends on its mode of stimulation (by NA or forskolin). Alternatively, interneurones may contain an additional subtype of adenylyl cyclase that is not activated by NA (but is nevertheless sensitive to forskolin), and which is Ca^{2+} insensitive.

Possible mechanisms of PKA-dependent enhancement of transmitter release

The present results indicate that the rate of mIPSCs is controlled by both cAMP and Ca^{2+}, and that the two internal messengers act synergistically. Many cellular processes are mediated by the co-operation of Ca^{2+} and cAMP, and each messenger influences the concentration of the other through complex regulatory loops (see Cohen, 1988, for review). In the present case, three main mechanisms may be proposed to account for the regulatory effects of PKA on transmitter release.

One possibility is that PKA modulates transmitter release by inducing Ca^{2+} release from Ca^{2+} stores. This seems unlikely because forskolin retained full effects after a long incubation in Ca^{2+}-free saline, which is likely to have depleted internal stores.

A second possibility is that PKA acts directly on the release process. Recently Yawo (1996) reported that NA enhances evoked transmitter release at the chick ciliary ganglion synapse. Ca^{2+} influx into the terminal is not involved for this modulation process. Yawo proposed that NA-dependent potentiation alters the Ca^{2+} sensitivity by phosphorylation of a protein close to the Ca^{2+} sensor of exocytosis. The same type of mechanism could apply here. However, the mechanisms underlying the regulation of mIPSCs in the present preparation need not be the same as those responsible for the regulation of evoked EPSCs in Yawo's work. One clear difference concerns the pharmacology of the NA receptors, which are of the β-type in the present preparation and which differ both from α- and β-types in the chick ciliary ganglion.

A third possibility would entail a change in the availability of releasing vesicles. Recent evidence indicates the existence of two pools in the nerve terminal, one reserve pool and the other for direct release (Pieribone, Shupliakov, Brodin, Hilfiker-Rothenfluh, Czernik & Greengard, 1995). Mice lacking synapsin, or lamprey axons injected with an antibody against synapsin, displayed a selective elimination of the vesicle pool distal to the active zone (Pieribone *et al.* 1995; Rosahl *et al.* 1995). These results indicate that synapsin controls the translocation of synaptic vesicles to the release site. In this context, synapsin could be proposed as a phosphorylated substrate for the PKA-dependent enhancement of transmitter release at cerebellar inhibitory synapses. However, whereas phosphorylation of synapsin by Ca^{2+}–calmodulin kinase controls the release of transmitter vesicles from the cytoskeleton (Llinas, Gruner, Sugimori, McGuinness & Greengard, 1991), no such role has been described so far for PKA-induced phosphorylation.

Capogna, M., Gähwiler, B. H. & Thompson, S. M. (1995). Presynaptic enhancement of inhibitory synaptic transmission by protein kinases A and C in the rat hippocampus *in vitro*. *Journal of Neuroscience* **15**, 1249–1260.

Chavez-Noriega, L. E. & Stevens, C. F. (1994). Increased transmitter release at exciatory synapses produced by direct activation of adenylate cyclase in rat hippocampal slices. *Journal of Neuroscience* **14**, 310–317.

Cohen, P. (1988). Protein phosphorylation and hormone action. *Proceedings of Royal Society of London* B **234**, 115–144.

Cooper, D. M. F., Mons, N. & Karpen, J. W. (1995). Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature* **374**, 421–424.

Gereau, R. W. & Conn, P. J. (1994). Presynaptic enhancement of excitatory synaptic transmission by β-adrenergic receptor activation. *Journal of Neurophysiology* **72**, 1438–1442.

Gray, R. & Johnston, D. (1987). Noradrenaline and β-adrenoreceptor agonists increase activity of voltage-dependent calcium channels in hippocampal neurons. *Nature* **327**, 620–622.

Greengard, P., Valtorta, F., Czernic, A. J. & Benfenati, F. (1993). Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* **259**, 780–785.

Hopkins, W. & Johnston, D. (1988). Noradrenergic enhancement of long-term potentiation of mossy fiber synapses in the hippocampus. *Journal of Neurophysiology* **59**, 667–687.

Huang, C.-C., Hsu, K.-S. & Gean, P.-W. (1996). Isoproterenol potentiates synaptic transmission primarily by enhancing presynaptic calcium influx via P- and Q-type calcium channels in the rat amygdala. *Journal of Neuroscience* **16**, 1026–1033.

Huang, Y.-Y. & Kandel, E. (1996). Modulation of both the early and the late phase of mossy fiber LTP by the activation of β-adrenergic receptors. *Neuron* **16**, 611–617.

Kaneko, A. & Tachibana, M. (1986). Blocking effects of cobalt and related ions on the γ-aminobutyric acid-induced current in turtle retinal cones. *Journal of Physiology* **373**, 463–479.

Lacaille, J. C. & Harley, C. W. (1985). The action of norepinephrine in the dentate gyrus: β-mediated facilitation of evoked potentials *in vitro*. *Brain Research* **358**, 210–20.

Lipscombe, D., Kongsamut, E. & Tsien, R. W. (1989). α-Adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type calcium-channel gating. *Nature* **340**, 639–642.

Llano, I. & Gerschenfeld, H. M. (1993*a*). Inhibitory synaptic currents in stellate cells of rat cerebellar slices. *Journal of Physiology* **468**, 177–200.

Llano, I. & Gerschenfeld, H. M. (1993*b*). β-Adrenergic enhancement of inhibitory synaptic activity in rat cerebellar stellate and Purkinje cells. *Journal of Physiology* **468**, 201–224.

Llinas, R., Gruner, J. A., Sugimori, M., McGuinness, T. L. & Greengard, P. (1991). Regulation by synapsin I and Ca^{2+}–calmodulin-dependent protein kinase II of transmitter release in squid giant synapse. *Journal of Physiology* **436**, 257–282.

Madison, D. V. & Nicoll, R. A. (1986). Cyclic adenosine 3′,5′-monophosphate mediates β-receptor actions or noradrenaline in rat hippocampal pyramidal cells. *Journal of Physiology* **372**, 245–259.

Pedarzani, P. & Storm, J. F. (1995). Protein kinase A-independent modulation of ion channels in the brain by cyclic AMP. *Proceedings of the National Academy of Sciences of the USA* **92**, 11716–11720.

Pieribone, V. A., Shupliakov, O., Brodin, L., Hilfiker-Rothenfluh, S., Czernik, A. J. & Greengard, P. (1995). Distinct pools of synaptic vesicles in neurotransmitter release. *Nature* **375**, 493–497.

ROSAHL, T. W., SPILLANE, D., MISSLER, M., HERZ, J., SELIG, D. K., WOLFF, J. R., HAMMER, R. E., MALENKA, R. C. & SÜDHOF, T. C. (1995). Essential functions of synapsins I and II in synaptic vesicle regulation. *Nature* **375**, 488–493.

RÜEGG, U. T. & BURGESS, G. M. (1989). Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends in Pharmacological Sciences* **10**, 218–220.

UNGERSTEDT, U. (1971). Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta Physiologica Scandinavica* **367**, 1–48.

WAYMAN, G. A., IMPEY, S., WU, Z., KINDSVOGEL, W., PRICHARD, L. & STORM, D. R. (1994). Synergistic regulation on the type I adenylyl cyclase by Ca^{2+} and G_s-coupled receptors *in vitro*. *Journal of Biological Chemistry* **269**, 25400–25405.

YAWO, H. (1996). Noradrenaline modulates transmitter release by enhancing the Ca^{2+} sensitivity of exocytosis in the chick ciliary presynaptic terminal. *Journal of Physiology* **493**, 385–391.

Acknowledgements

We would like to thank Dr H. M. Gerschenfeld and Dr I. Llano for providing some of the results used in Fig. 4 and, together with Dr C. Rosenmund, for useful discussions as well as critical and helpful comments on the manuscript. We also thank Dr P. Vincent and Mr C. Pouzat for the programs used for analysis of synaptic activity. We wish to thank the Bristol-Myers Squibb Corporation for the gift of SQ 22,536. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 406) and from the European Community (ERBCIPACT 92-3014 and CHRX-CT94-0500) to A. M., and a Human Frontier Science Program long-term fellowship (LT381/95) to S. K.

Author's email address

S. Kondo: skondo@gwdg.de

Received 25 July 1996; accepted 25 September 1996.